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Mast cells generate cysteinyl leukotrienes and interferon-β as well as evince impaired IgE-dependent degranulation upon TLR7 engagement

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Mast cells are numerous at anatomical sites close to external environment, virtually at the portals of infection. A few data indicated that these cells express cytoplasmic Toll-like receptors (TLRs) recognizing virus-derived molecules. Accordingly, mast cells could participate in anti-viral defense or/and in viral-related diseases. However, data concerning the influence of viruses on mast cell activity are limited. Thus, the aim of our study was to determine mast cell response to TLR7 ligand, i.e. resiquimod (R848), a synthetic mimic of viral ssRNA. Since mast cells play a central role in allergic reactions the effect of TLR7 agonist was also investigated on FcεRI-dependent mast cell response. Experiments were carried out *in vitro* on freshly isolated fully mature rat peritoneal mast cells. Mast cells exhibit constitutive TLR7 molecule expression and its up-regulation after the agonist challenge. TLR7-mediated mast cell stimulation resulted in cysteinyl leukotriene (cysLT) and interferon (IFN)-β synthesis, whereas no histamine and CXCL8 secretion was stated. Moreover, mast cell priming with TLR7 ligand caused the reduction in anti-IgE-induced histamine release. The results suggest that ssRNA viruses could directly activate mast cells to alter their phenotype and to release of potent proinflammatory mediators or indirectly modulate IgE-dependent allergic processes.

Keywords: Interferons, Leukotrienes, Mast cells, Toll-like receptor 7, Viral infection

Mast cells are long-lived resident tissue cells widely distributed throughout the body. These cells are capable to secrete a broad range of mediators with diverse biological effects[1-4]. Thus, mast cells may play a vital role in maintaining homeostasis and in various physiological processes, although they are also involved in many pathological events[1-3]. Moreover, it is well established that mast cells participate in a wide range of inflammatory conditions[5,6], including IgE-mediated allergic reactions[5,7].

Nowadays, a growing body of evidence indicates that mast cells represent a critical component of host defense, especially in the early clearance of bacterial infections[8]. These cells are particularly numerous in the skin, mucosal surfaces, and in the vicinity of blood vessels, virtually at the sites of bacteria entry[1-4]. Mast cells also express pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs)[9], by which they detect invading bacteria. These cells have the ability to kill bacteria *via* production and release of antimicrobial peptides, including defensins and cathelicidins[10,11]. Further, mast cells are capable to phagocytose and subsequently eliminate microbes by oxidative and non-oxidative mechanisms[12]. Interestingly, these cells may entrap bacteria in extracellular structures and kill them independently of phagocytosis[13]. Another significant mast cell function in antibacterial defense is to elicit the development of inflammation. Mast cells activated *via* TLR2- and/or TLR4-specific bacterial ligands produce cysteinyl leukotrienes (cysLTs)[14-19] that are particularly potent in initiating and sustaining the inflammatory response[20]. In addition, bacteria and bacterial antigens or toxins may stimulate mast cells to release of other important proinflammatory humoral factors, including tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and chemokines CCL2, CCL3, CCL20, and CXCL8[21-27]. Moreover, there is some evidence that mast cells present bacterial antigens in the context of class I and II molecules of major histocompatibility complex (MHC) *in vitro*[28,29] as well as, in the context of class I MHC, *in vivo*[30] and promote the development of adaptive immunity.

There are some data indicating that mast cells express virus-specific PRRs, primarily endosomal TLR3, TLR7, TLR8 and TLR9[9,31-34]. Thus, it might be

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assumed that these cells can respond to viruses and thereby participate in the mechanisms of antiviral immunity and/or pathomechanisms of viral-related diseases. However, data concerning the influence of viruses on mast cell activity are limited. Among the intracellular receptors, TLR7 seems to be essential in viral infections as it mediates the response to uridine-rich single-stranded RNA (ssRNA), a genomic sequence of the widespread viruses, including influenza virus or human immunodeficiency virus (HIV)[35]. Therefore, the aim of our study is to determine whether resiquimod (R848), a synthetic mimic of viral ssRNA, may induce degranulation and preformed mediator release from mature mast cells. We also examined if R848 stimulates mast cells to *de novo* cysLTs, CXCL8 and interferon β (IFN-β) generation. Since mast cells play a central role in allergic reactions[5,7], we also investigated the effect of TLR7 agonist on FcεRI-dependent mast cell response.

Materials and Methods

Animals—Female albino Wistar rats weighting 200-250 g, aged 3-4 months were used. They were bred in animal quarters of the Medical University of Łódź in the standard storage conditions, i.e. artificial lighting for 12 h and 12 h of darkness, room temperature (20 ± 2 °C), in metal cages, 5 rats in each. The animals were provided with LSM Murigran granulated fodder for rodents and water *ad libitum.* The experimental procedures were approved by the Local Ethics Committee for Experiments on Animals of the Medical University of Łódź (the approval No. 25/ŁB 609/2012).

Mast cell isolation—Mast cells were isolated from peritoneal cavities by lavage with 50 mL of 1% Hank's Balanced Salt Solution (HBSS) with the addition of 0.015% $NaHCO_3$ (Life Technologies, Gaithersburg, MD, USA). Next, after the abdominal massage (90 sec), the cell suspension was aspirated from the peritoneal cavity, centrifuged (150 *g*, 5 min, 20 °C), and washed twice in complete Dulbecco's Modified Eagle Medium (cDMEM) containing DMEM supplemented with 10 µg/mL gentamicin, 10% fetal calf serum (FCS) and 2 mM glutamine (150 *g*, 5 min, 20 °C) (Life Technologies). To obtain purified mast cells, peritoneal cells were resuspended in isotonic 72.5% Percoll (Sigma-Aldrich Co. St. Louis, MO, USA) density gradient and centrifuged (190 *g*, 15 min, 20 °C). Subsequently, mast cells were centrifuged twice in cDMEM (150 *g*, 5 min, 20 °C), counted and resuspended in a suitable volume of medium for rat mast cells containing 2.7 mM KCL, 1 mM $MgCl_2$, 137 mM NaCl, 1 mM $CaCl_2$, 10 mM N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid (HEPES) buffer, 1 mg/mL bovine serum albumin (BSA) and 5.6 mM glucose (Sigma-Aldrich) (the *p*H of the medium was adjusted to 6.9) to obtain mast cell concentration of 1.5×10^6 cells/mL. Mast cells purity established by metachromatic staining with toluidine blue (Sigma-Aldrich) was over 98%.

Western blot analysis—Purified mast cells were suspended in the medium and incubated with medium alone (constitutive TLR7 expression) or R848 at final concentrations of 100 µg/mL for 6 h at 37 °C in a humidified atmosphere with 5% CO_2. For positive control of constitutive TLR7 expression human peripheral blood-derived mononuclear cells (hPBMCs) prepared by Ficoll separation[36] was applied. The cell lysis was conducted in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, *p*H 8.0, with 1% Igepal CA-630 (NP-40), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS) and 0.5% sodium deoxycholate (Invivogen, San Diego, CA, USA)) containing protease inhibitor cocktail (1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 50 µM bestatin, 800 nM aprotinin, 15 µM E64, 10 µM pepstatin A, 20 µM leupeptin, 5 mM ethylenediaminetetraacetic acid (EDTA)) (Thermo Scientific, Rockford, IL, USA) over 30 min on ice and undissolved residues were removed. Next, the protein concentration in the lysates were analyzed using Bradford Assay (Bio-Rad Laboratiories, Inc., United States). The cell lysates (50 µg) were separated on NuPAGE 10% Bis-Tris Gel (Life Technologies) prior to transfer to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). TLR7 expression was determined by rabbit anti-TLR7 antibodies (1:500 dilution) cross-reactive with human and rat TLR7, followed by addition of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000 dilution) (LifeSpan Biosciences, Inc., Seatle, WA, USA). TLR7 protein was visualized by enhanced chemiluminescence (ECL) system using the application of ECL Chemiluminescent Substrate Reagent Kit (Life Technologies) according to manufacturer's instructions. To confirm equivalent protein load per well, the membranes were reprobed with rabbit anti-β-actin antibody cross-reactive with human and rat β-actin (Abnova Corporation, Taipei, Taiwan).

Histamine release assay—For histamine release assay, medium-suspended purified mast cells were divided into 90 μL aliquots and 10 μL of R848 (InvivoGen, San Diego, CA, USA) at final concentrations of 0.1, 1, 10 or 100 μg/mL, compound 48/80 (Sigma-Aldrich) at final concentration of 5 μg/mL (positive control) or medium alone (negative control) was added. Next, aliquots were incubated for 1 h or 1.5 h at 37 °C in water bath with constant stirring. To stop the reaction, 1.9 mL of cold medium was added. Subsequently, centrifugation (253 *g*, 5 min, 4 °C) of the cell suspensions was performed and the supernatants were decanted into individual tubes. Then, 2 mL of distilled water was added to each tube containing cell pellet. The samples were acidified with 3 N HCL. The histamine content was determined in both cell pellets (residual histamine) and supernatants (released histamine) by spectrofluorometric method using o-phthaldialdehyde (OPT) (Sigma-Aldrich)[37] (the excitation wavelength was 360 nm, while the fluorescence wavelength was 450 nm). Histamine release was expressed as a percentage of the total cellular content of the amine. In separate experiments mast cells were preincubated with R848 at final concentrations of 0.1 μg/mL or 100 μg/mL or medium alone for 1 h at 37 °C. After rinsing, mast cells were challenged with anti-IgE (Serotec, Oxford, UK) at final concentration of 5 μg/mL or medium alone (negative control) for 30 min at 37 °C. The reaction was stopped by the cold medium and histamine content was measured, as described above.

CysLT release assay—Purified mast cells were suspended in the medium and incubated with R848 at final concentrations of 0.1 μg/mL or 100 μg/mL, calcium ionophore A23187 (Sigma-Aldrich) at final concentration of 5 μg/mL (positive control) or medium alone (negative control) for 2 h or 3 h at 37 °C. Centrifugation (150 *g*, 5 min, 20 °C) was performed to collect the supernatants which were then analyzed by ELISA commercial kit (Cayman Chemical, Ann Arbor, MI, USA) detecting LTC_4 and its degradation products LTD_4 and LTE_4. The assay sensitivity was < 13 pg/mL. In separate experiments mast cells were treated with anti-IgE at final concentration of 5 μg/mL, R848 at final concentrations of 0.1 μg/mL or 100 μg/mL, both R848 and anti-IgE, or medium alone (negative control) for 2 h at 37 °C. Mast cell suspensions were centrifuged to collect supernatants which were then analyzed for cysLT synthesis, as described above.

CXCL8 and IFN-β release assays—Purified mast cells were suspended in the medium and incubated with R848 at final concentrations of 0.1 μg/mL or 100 μg/mL, medium alone (negative control), or anti-IgE at final concentration of 5 μg/mL for 6 h at 37 °C in a humidified atmosphere with 5% CO_2. Centrifugation (150 *g*, 5 min, 20 °C) was performed to collect the supernatants which were then analyzed by ELISA commercial kit (Wuhan EIAAB Science CO. LTD, Wuhan, China) detecting CXCL8 or IFN-β; the sensitivity of the assays was < 7.8 pg/mL or < 2.7 pg/mL, respectively. In separate experiments mast cells were pretreated with R848 at final concentrations of 0.1 μg/mL or 100 μg/mL, or medium alone for 1 h at 37 °C. After rinsing, mast cells were stimulated with anti-IgE at final concentration of 5 μg/mL or medium alone (negative control) for 6 h at 37 °C in a humidified atmosphere with 5% CO_2. Mast cell suspensions were centrifuged to collect supernatants which were then analyzed for CXCL8, as described above.

TLR7 blocking experiments—To establish the specificity of R848 action, mast cells were pretreated with goat polyclonal anti-TLR7 IgG antibodies or goat IgG isotype control (Santa Cruz Biotechnology) at final concentration of 40 μg/mL or medium alone (negative control) for 1 h at 37 °C, before main procedure accomplishments (i.e. cysLT and IFN-β synthesis assays).

Statistical analysis—Statistical analysis involved mean value, standard deviation (SD) and Student's *t*-test for small groups. $P<0.05$ values were regarded as statistically significant.

Results

Mast cell TLR7 expression— To our knowledge, mature tissue rat mast cells have not yet been reported for TLR7 protein expression. Thereby, initially these cells were investigated for TLR7 molecule. The western blot analysis demonstrated mast cell constitutive expression of TLR7 protein comparable to this receptor expression in PBMCs. In addition, mast cell exposure to R848 at the concentration of 100 μg/mL resulted in a pronounced up-regulation of TLR7 protein expression (Fig.1).

The effect of R848 on mast cell performed mediator release—First the effect of TLR7 ligand R848 was examined on mast cell degranulation and preformed

mediator release. The entire range of R848 concentrations used did not trigger mast cell degranulation and histamine secretion (Fig. 2). In comparison, a potent degranulation inducer, i.e. compound 48/80, induced mast cell histamine release up to 65.8 ± 3.8% (mean ± SD) upon 1.5 h of incubation.

The effect of R848 on mast cell newly generated mediator release—Next, we investigated whether R848-activated mast cells generate *de novo* synthesized mediators. It was found that TLR7 ligand stimulated mast cells to cysLT generation (Fig. 3). R848 at the concentration of 0.1 µg/mL activated mast cells to release up to 213.4 ± 25.2 and up to 302.2 ± 28.7 pg cysLT/1.5×10^6 mast cells after 2 h and 3 h of incubation, respectively. CysLT secretion in response to mast cell stimulation with R848 used at 100 µg/mL was up to 258.0 ± 29.1 pg/1.5×10^6 mast cells (exposure time – 2 h) and 382.3 ± 42.6 pg/1.5×10^6 mast cells (exposure time – 3 h). In comparison, at the same experimental conditions mast cells treated with calcium ionophore A23187 released up to 921.7 ± 49.9 pg/1.5×10^6 mast cells (2 h of stimulation) and 950.0 ± 42.4 pg/1.5×10^6 mast cells (3 h of stimulation) of cysLTs. In order to analyze the specificity of R848 action on mast cell cysLT synthesis and release, blocking experiments were carried out using anti-TLR7 antibodies. Pretreatment of mast cells with these antibodies almost completely inhibited R848-induced cysLT secretion. Mast cell cysLT generation in response to R848 was not affected by isotype control antibodies (Fig. 3).

Mast cell stimulation with TLR7 ligand resulted in a significant generation and release of IFN-β, but not CXCL8 (Fig. 4). Mast cells secreted up to 108.2 ± 63.1 and 497.4 ± 113.7 pg IFN-β/1.5×10^6 mast cells upon 6 h-treatment with R848 at the final concentrations of 0.1 and 100 µg/mL, respectively. At the same experimental conditions, in response to anti-IgE challenge, mast cells did not synthesize and release IFN-β, but secreted CXCL8 (up to 410.1 ± 55.6 pg/1.5×10^6 mast cells). R848-induced IFN-β secretion was strongly reduced by mast cell preincubation with anti-TLR7 blocking antibodies. Mast cell IFN-β generation in response to R848 was not affected by isotype control antibodies (Fig. 4).

The effect of R848 on mast cell IgE-mediated mediator release—Subsequently, whether R848

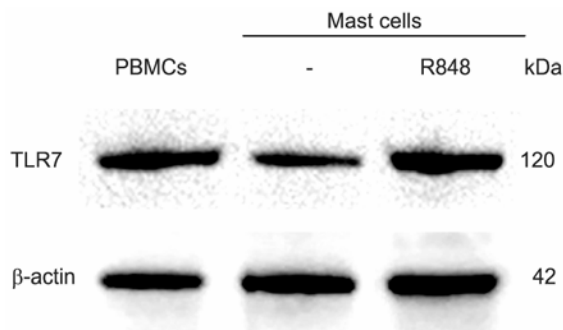

Fig. 1—TLR7 protein expression in rat peritoneal mast cells. Mast cells were treated with medium alone (constitutive expression) or R848 at 100 µg/mL for 6 h. The constitutive TLR7 molecule expression in hPBMCs is presented as a positive control. β-actin was used as loading control. Results are representative of 3 independent experiments.

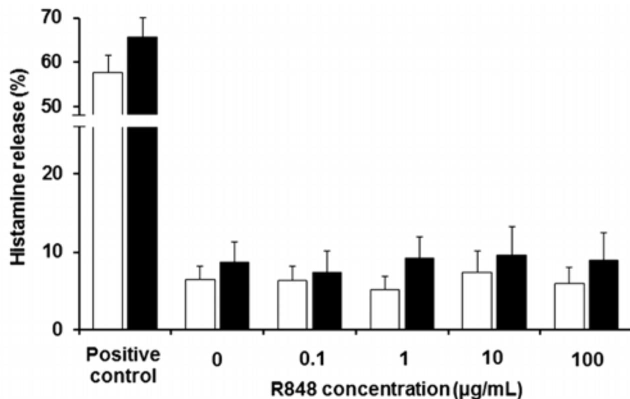

Fig. 2—TLR7 ligand R848-induced histamine release from mast cells. Mast cells were incubated with R848, compound 48/80 (positive control) or medium alone (negative control) for 1 h (□) or 1.5 h (■). Results are the mean ± SD of three independent experiments and each experiment was done in duplicate (n=6).

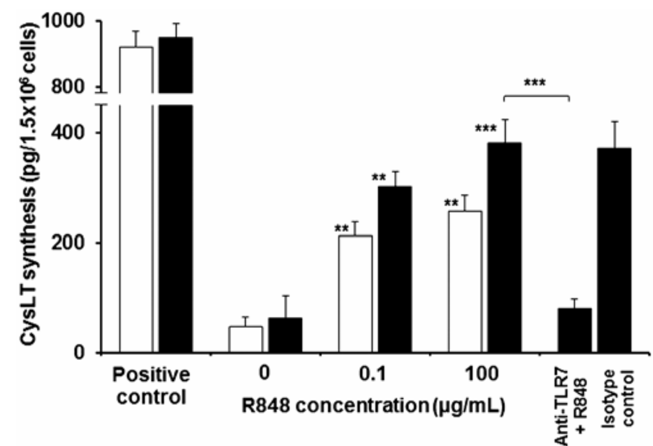

Fig. 3—TLR7 ligand R848-induced mast cell cysLT generation. Mast cells were incubated with R848, calcium ionophore A23187 (positive control) or medium alone (negative control) for 2 h (□) or 3 h (■), or pretreated with anti-TLR7 or isotype control antibodies for 1 h before the addition of R848. Results are the mean ± SD of three independent experiments and each experiment was done in duplicate (n=6). ** P<0.01; *** P<0.001.

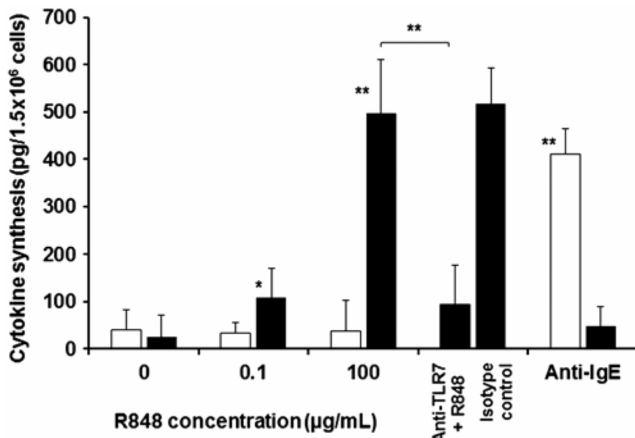

Fig. 4—TLR7 ligand R848-induced mast cell CXCL8 (□) and IFN-β generation (■). Mast cells were incubated with R848, anti-IgE or medium alone (negative control) for 6 h, or pretreated with anti-TLR7 or isotype control antibodies for 1 h before the addition of R848. Results are the mean ± SD of three independent experiments and each experiment was done in duplicate (n=6). * $P<0.05$; ** $P<0.01$.

influence an IgE-dependent mediator release from mast cells was investigated. As shown in Fig. 5A, mast cells primed for 1 h with TLR7 agonist at the concentration of 100 µg/mL showed a significant ($P<0.05$) decrease in anti-IgE-induced histamine secretion. Pretreatment of mast cells with R848 did not affect cysLT synthesis triggered by anti-IgE (Fig. 5B). Similarly, mast cell priming with TLR7 ligand at concentrations of 0.1 and 100 µg/mL for 1 h did not modulate anti-IgE-induced CXCL8 release (Fig. 5C).

Discussion

It is well known that mast cells are mainly located close to the external environment, in fact at the portals of infection. Moreover, these cells are the potential source of multiple mediators and cytokines, including TNF, IL-1β, IL-6, IL-12, IL-18 and IL-33, various chemokines, as well as type I and type II interferons (IFNs), that might be involved in the mechanisms of antiviral defense[1-4]. These mediators can promote the development of inflammation and influence the activity of NK cells, $CD8^+$ T lymphocytes, dendritic cells, $CD4^+$ T lymphocytes, and B cells, i.e. cell populations participating in the innate and adaptive immune response to viruses[3,4,7,38]. What is more, various mast cell-derived chemokines, such as CCL3, CCL4, CCL5, CXCL1, CXCL8 and CXCL12 attract $CD8^+$ T lymphocytes and/or NK cells to the site of virus infection.

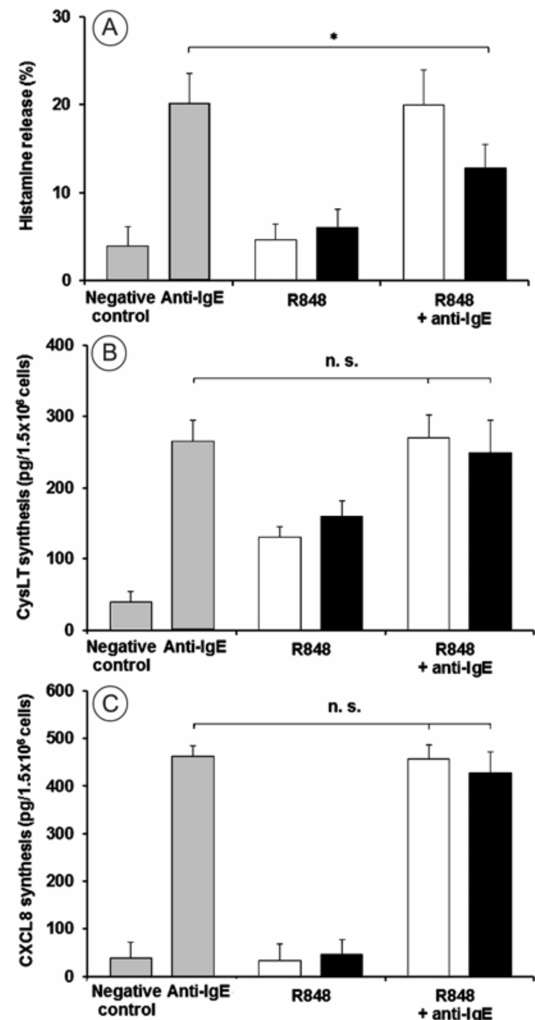

Fig. 5—Effect of TLR7 ligand R848 on IgE-mediated (A) histamine release, (B) cysLT generation and (C) CXCL8 synthesis by mast cells. (A) Mast cells were preincubated with R848 at concentrations of 0.1 µg/mL (□) or 100 µg/mL (■) or medium alone for 1 h and then incubated with anti-IgE or medium (negative control) for 30 min; (B) Mast cells were exposed to R848 at concentrations of 0.1 µg/mL (□) or 100 µg/mL (■), anti-IgE, medium alone (negative control), or both R848 and anti-IgE for 2 h; (C) Mast cells were pretreated with R848 at concentrations of 0.1 µg/mL (□) or 100 µg/mL (■) or medium alone for 1 h and then incubated with anti-IgE or medium (negative control) for 6 h. Results are expressed as the mean ± SD of three separate experiments and each experiment was done in duplicate (n=6). * $P<0.05$.

It is commonly known that viral genomic nucleic acids serve as viral pathogen-associated molecular patterns (PAMPs) for endosomal TLRs, while viral replication intermediates and by-products are recognized by cytosolic sensors[39]. Recently, mast cells have been documented to express endosomal and cytosolic virus-specific PRRs, mainly intracellular

TLRs[9,31-34], but also RIG-I-like receptors (RLRs), such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)[40]. Different mast cell lines express TLR3 mRNA[31-34,41] and TLR3 protein[31,34,41]. The expression of TLR9 was shown in several mast cell lines at both the transcript and protein level[31-34,42]. TLR8 mRNA was documented only in murine bone marrow-derived mast cells (BMMCs)[31]. Immature human mast cell lines[31,33,34] as well as mature murine fetal skin-derived cultured mast cells (FSMCs)[33] and human skin and lung mast cells[31] were shown to express TLR7 transcript, whereas TLR7 protein was detected only in immature mast cells[31,34]. In the present study connective tissue mast cells freshly isolated from rat peritoneal cavity showed the constitutive expression of TLR7 molecule. Therefore, these cells might have the ability to sense and respond to viruses and/or their products.

There is some evidence of mast cell activation by TLR3 ligand, a synthetic mimic of viral double-stranded (ds)RNA, i.e. polyriboinosinic:polyribocytidilic acid (poly(I:C)). TLR3-stimulated mast cells secreted a diverse array of chemokines[33,41,43], IFN-α, TNF, IL-6[31,33] and a small amount of cysLTs[31]. Furthermore, Orinska *et al.*[41] documented that TLR3 ligation affected mast cell phenotype by altering the expression of some cell surface molecules. Also, little is known about TLR9-mediated mast cell activation, upon which the release of TNF, IL-1β, IL-6, CCL3, CCL4, CCL5 and cysLTs has been only stated[33,44,45]. According to our knowledge, mast cell response to TLR8 ligation has not yet been examined.

With regard to studies on mast cell TLR7-mediated stimulation, as far as we know, it has been hitherto only two reports. Yang *et al.*[34] reported that TLR7 ligand activated P815 mast cell line to IL-6 secretion. Matsushima *et al.*[33] indicated that R848 did not induce FSMC and BMMC degranulation, but activated FSMCs to a dose-dependent production of TNF, IL-6, CCL3, CCL4, and CCL5.

In the present study rat mast cell stimulation *via* TLR7 up-regulated TLR7 protein expression and induced cysLT and IFN-β secretion, but did not activate these cells to degranulation and preformed mediator release or to *de novo* CXCL8 synthesis. Moreover, the observed effects of mast cell cysLT and IFN-β generation were clearly specific to the interaction between TLR7 and its agonist, as TLR7 blocking antibody strongly inhibited the synthesis of these mediators. However, the amount of released cysLTs was relatively small, it should be noted that these lipid mediators are extremely potent proinflammatory factors exerting physiological effects at concentrations even as low as nanomolar[20].

It is well known that viral infections entail exacerbation of allergic diseases, particularly bronchial asthma[46], but the cause of this phenomenon remains unknown. It might be therefore assumed that TLR-specific viral ligands can modulate mast cell responsibility in IgE-mediated allergic reactions. Saluja *et al.*[47] indicated that prolonged (96 h) exposure to poly(I:C) significantly augmented mast cell IgE-mediated degranulation and LT production. In addition, as shown by Kulka *et al.*[31], TLR3 agonist induced augmentation of cysLT as well as proinflammatory cytokin release by antigen-stimulated IgE-sensitized mast cells. On the other hand, the same authors demonstrated that TLR3 ligation decreased mast cell adhesion to extracellular matrix proteins and, consequently, impaired their IgE-mediated degranulation[32]. In the present study it was established that TLR7 ligand R848 had no effect on IgE-dependent generation of cysLTs and CXCL8. However, mast cell priming for 1 h with ssRNA analogue at high concentration induced a significant extenuation of mast cell IgE-mediated degranulation and preformed mediator release.

In conclusion, the present observations may imply that mast cells can be directly activated to alter their phenotype and to selectively secrete proinflammatory mediators during ssRNA virus infections. In addition, the results showing that priming with TLR7 ligand affects FcεRI-mediated mast cell response suggest that ssRNA virus infection can modulate mast cell reactivity in IgE-dependent allergic reactions. Nonetheless, the present findings together with those of Matsushima *et al.*[33] and Yang *et al.*[34] are far from sufficient to clarify the effect of TLR7-mediated activation on mast cell response and its consequent biological implications, thus further studies are necessary.

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